REMARKS

Claims 1, 3-9 and 11-21 are pending. Claims 2 and 10 have been cancelled. Claims 6, 14 and 18-21 are withdrawn from consideration. Claims 1, 3-9 and 11-21 have been amended to more clearly reflect the claimed invention by limiting the step of imparting antigen specificity. Support for the amendment on these claims can be found in the specification on pages 5 to 9 and in the original claims. Claims 7 and 15 are amended to correct their dependency. No new matter has been added.

Pending claims 1, 3-5, 7-9, 11-13 and 15-17 considered together with the following remarks are believed sufficient to place the application into condition for allowance. Accordingly, early and favorable action on the merits are earnestly solicited.

Election/Restriction

The Examiner has required election in the present application between:

Group I, claims 1-5, 7-13 and 15-17, drawn to a process of preparing cells for cell therapy comprising inducing Th cells or both Th1 and Tc1 cells that having a nonspecific antitumor activity and imparting antigen specificity to the cells by transducing a gene for a TCR that recognize a cancer-associated antigen;

Group II, claims 1, 6-9 and 14-17, drawn to a process of preparing cells for cell therapy comprising inducing Th cells or both Th1 and Tc1 cells that having a nonspecific antitumor activity and imparting antigen specificity to the cells by culturing a T cell-containing material in the presence of anti-CD3 antibody and IL-2, or further includes IL-12;

Group III, claims 18 and 19, drawn to cells for cell therapy that are produced by a process comprising inducing Th cells or both Tc1 and Th1 cells that having a nonspecific antitumor activity and imparting antigen specificity to the cells; and

Group IV, claims 20 and 21, drawn to a method for preventing or treating tumor by using the cells of group III.

Applicants herein reaffirm the election, with traverse, Group I, Claims 1-5, 7-13, and 15-17.

It is again submitted that at least groups I and II should be examined together in view of the common technical features as reflected, for example, in claim 1 which is generic as to both groups.

The Examiner has also required an election in the present application between the species listed below:

Cancer-associated antigen WT1, CEA, AFP, CA19-9, CA125, PSA, CA72-4, SCC, MK-1, MUC-1, p53, HER2, G250, gp-100, MAGE, BAGE, SART, MART, MYCN, BCR-ABL, TRP, LAGE, GAGE, and NY-ESO1.

Applicants herein reaffirm the election of Species WT1. Once the elected species is found to be allowable, the Examiner is requested to expand the examination of the claims to the non-elected species.

All of the elected claims listed above (claims 1-5, 7-13 and 15-17) are directed to the elected species. As acknowledged by the Examiner, at least claims 1-4, 7-12 and 15-17 are generic.

Claim Objection

The Examiner has objected to claims 7-8 and 15-17 under 37 CFR 1.75(C) as being in improper form because a multiple dependent claim shall not serve as a basis for any other multiple dependent claim.

In response to this objection, claims 7 and 15 have been amended to correct this deficiency.

Reconsideration and withdrawal of the above objection are respectfully requested.

Rejection under 35 U.S.C. § 112, second paragraph

The Examiner has rejected claims 1-5, 7-13 and 15-17 under 35 U.S.C. § 112, second paragraph, as being indefinite for reciting the use of abbreviations.

In response to this rejection, claims 1-5, 7-13 and 15-17 have been amended to correct this deficiency, replacing the abbreviations recited in the claims with non-abbreviated terms.

Claims 2 and 10 have been cancelled herein without prejudice or disclaimer, thus obviating the rejection as to these claims. As to the terms "class I-restricted" and "class II-restricted", Applicants traverse the basis for this rejection. Applicants argue that these terms are well known in the art. Applicants have provided a copy of relevant pages of a standard textbook "therapeutic Immunology" and Phan *et al.* (2003), both demonstrating that immunization of patients using class-I and class-II-restricted peptides are well known in the art. Applicants contend that the terms "class I-restricted" and "class II-restricted" are based on the description of the specification and/or a knowledge well-known in the art.

Reconsideration and withdrawal of the above rejection are respectfully requested.

Rejection under 35 U.S.C. § 102 (b)

The Examiner has rejected claim 1 under 35 U.S.C. § 102(b) as being anticipated by Ohmi *et al.*, 1999 (Cancer Immunology, Immunotherapy, Vol. 48, p. 456-462).

In response to this rejection, claim 1 has been amended to limit the step of imparting antigen specificity.

Accordingly, Ohmi *et al.* do not teach the amended limitation of imparting antigen specificity, but is drawn to inducing helper T cells having a nonspecific antitumor activity. Therefore, Ohmi *et al.* do not disclose all of the limitations of the presently claimed invention. Anticipation requires that "each and every element as set forth in the claim is found, either

expressly or inherently described, in a single prior art reference." (*See*, *In re Robertson*, 169 F.3d 743, 745, 49 U.S.P.Q.2d 1949 (Fed. Cir. 1990), quoting *Verdegaal Bros., Inc. v. Union Oil Co.*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987)).

Reconsideration and withdrawal of the anticipation rejection of claim 1 are respectfully requested.

Rejection under 35 U.S.C. § 103(a)

The Examiner has rejected claims 1-5, 7-13 and 15-17 under 35 U.S.C. § 103(a) as being unpatentable over Tsuji *et al.* April 2003 (Cancer Science, Vol 94, No.4, p. 389-393) in view of Gaiger *et al.* 2008 (US Patent No. 7,323,181) and Nishimura, Takashi, 2000 (Cancer Treatment and Host, Vol. 12, No. 4, p. 363-373, IDS-CL).

The present invention is drawn to a process of preparing cells for cell therapy, comprising the steps of:

inducing helper T cells that have a nonspecific antitumor activity; and

imparting antigen specificity to the helper T cells

wherein the step of imparting antigen specificity to the helper T cells is carried out by transducing a T cell receptor gene that recognizes a cancer-associated antigen as recited for example in claim 1.

Tsuji discloses a method for preparing antigen-specific cytoxic T cells by introducing a T Cell receptor gene, while the present invention features antigen-specific helper T cells. Tsuji does not teach or suggest the advantage of using helper T cells in the claimed process.

It is well established in the art that cytoxic T cells and helper T cells are completely different in terms of its origin, recognition properties of MHC molecules, and function in stimulating the immune system. The function of a T cell receptor is not only to bind with the target antigen (the antigen peptide bound to MHC), but also to transduce the signal for T cell stimulation. Also, it is well known in the art, cytoxic T cells comprise a CD8 molecule and a complex of a T cell receptor and CD3, while helper T cells comprise a CD4 molecule and a complex of a T cell receptor and CD3. In order to transduce the signal for stimulating T cells,

CD8 (for cytoxic T cells) or CD4 (for helper T cells) and a complex of a T cell receptor and CD3 antigen have to bind with the target antigen (the antigen peptide bound to MHC) and transmit the signal to T cells.

Namely, all of the following elements are essential for the T cell receptor function:

for cytoxic T cells: (MHC Class 1 + peptide) - (T cell receptor +CD3+CD8)

for helper T cells: (MHC Class II + peptide) - (T cell receptor +CD3+CD4)

Since such a signal transduction is required for cytoxic T cells or helper T cells to exert its function, one cannot expect or predict from the disclosure of the cytoxic T cell of Tsuji whether helper T cells containing a T cell receptor gene indeed exhibit an anti-tumor activity. A person skilled in the art could not predict whether the helper T cells are able to function in an antigen-specific manner unless one skilled in the art actually conducts this experimentation.

Furthermore, it was even more unpredictable in the cases of atypical combinations such as:

(MHC Class I + peptide) - (T cell receptor +CD3+CD4)

(MHC Class II + peptide) - (T cell receptor +CD3+CD8)

Thus, a person skilled in the art could not have conceived of the present invention based on the disclosure of Tsuji.

Nishimura is a review article related to helper T1/T2 cells. The Nishimura reference only discloses that there are two types of helper T cells, and that helper T1 cells appear to play a relatively more important role than helper T2 cells. However, it fails to teach or suggest how one can achieve a helper T1-dominant immune system.

Accordingly, a person skilled in the art could not reasonably expect that antigen-specific helper T cells can be obtained by introducing the T cell receptor gene into helper T cells from the combination of Tsuji (which discloses cytotoxic T cells) and Nishimura (which is a review article about helper T1/T2 cells).

Gaiger merely discloses the cancer antigen Wilms' Tumor 1 (WT1). As discussed above, a person skilled in the art could not predict whether helper T cells with the T cell receptor gene can function based on the discloser of Tsuji and Nishimura.

Accordingly, a person skilled in the art could not achieve the present invention based on Tsuji in view of Gaiger and Nishimura.

Reconsideration and withdrawal of the obviousness rejection of claims 1-5, 7-13 and 15-17 are respectfully requested.

CONCLUSION

In view of the above amendment, applicant believes the pending application is in condition for allowance.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Eggerton Campbell Reg. No. 51307 at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37.C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Dated: FEB 17 2009

Respectfully submitted,

By _____
Gerald M. Murphy, Jr.
Registration No.: 28,977
BIRCH, STEWART, KOLASCH & BIRCH, LLP
8110 Gatehouse Road
Suite 100 East
P.O. Box 747
Falls Church, Virginia 22040-0747
(703) 205-8000
Attorney for Applicant

GMM/EAC/kmlMM/EAC

EAC

Journal of Immunotherapy
26(4):349–356 © 2003 Lippincott Williams & Wilkins, Inc., Philadelphia

Immunization of Patients with Metastatic Melanoma Using Both Class I- and Class II-Restricted Peptides from Melanoma-Associated Antigens

Giao Q. Phan, Christopher E. Touloukian, James C. Yang, Nicholas P. Restifo, Richard M. Sherry, Patrick Hwu, Suzanne L. Topalian, Douglas J. Schwartzentruber, Claudia A. Seipp, Linda J. Freezer, Kathleen E. Morton, Sharon A. Mavroukakis, Donald E. White, and Steven A. Rosenberg

Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD

Summary: Cancer vaccines targeting $CD8^+$ T cells have been successful in eliciting immunologic responses but disappointing in inducing clinical responses. Strong evidence supports the importance of $CD4^+$ T cells in "helping" cytotoxic $CD8^+$ cells in antitumor immunity. We report here on two consecutive clinical trials evaluating the impact of immunization with both human leukocyte antigen class I- and class II-restricted peptides from the gp100 melanoma antigen. In Protocol 1, 22 patients with metastatic melanoma were immunized with two modified class I A*0201-restricted peptides, gp100:209-217(210M) and MART-1:26-35(27L). In Protocol 2, 19 patients received the same class I-restricted peptides in combination with a class II DRB1*0401-restricted peptide, gp100:44-59. As assessed by in vitro sensitization assays using peripheral blood mononuclear cells (PBMC) against the native gp100:209-217 peptide, 95% of patients in Protocol 1 were successfully immunized after two vaccinations in contrast to 50% of patients in Protocol 2 ($P_2 < 0.005$). Furthermore, the degree of sensitization was significantly lower in patients in Protocol 2 ($P = 0.01$). Clinically, one patient in Protocol 2 had an objective response, and none did in Protocol 1. Thus, the addition of the class II-restricted peptide gp100:44-59 did not improve clinical response but might have diminished the immunologic response of circulating PBMC to the class I-restricted peptide gp100:209-217. The reasons for this decreased immune reactivity are unclear but may involve increased $CD4^+CD25^+$ regulatory T-cell activity, increased apoptosis of activated $CD8^+$ T cells, or the trafficking of sensitized $CD8^+$ reactive cells out of the peripheral blood. Moreover, the sequential, nonrandomized nature of patient enrollment for the two trials may account for the differences in immunologic response. **Key Words:** gp100, MART-1, $CD4^+$ T cell, $CD8^+$ T cell, $CD4^+CD25^+$ T cell

The 5-year survival of patients with metastatic melanoma is less than 2% in most published series.[1] Treatment options for these patients are limited. Chemotherapy can induce transient responses but is rarely, if ever, curative. Immunotherapy with high-dose intravenous interleukin-2 (IV IL-2) has been associated with durable, and probably curative, responses,[2–4] but the overall response rate is only 16%.[5] The identification of tumor-associated antigens and the ability to manufacture pharmaceutical-grade peptides have led to vaccination trials evaluating the extent to which immunotherapy can be used for the treatment of patients with metastatic

Received January 6, 2003; accepted March 6, 2003.
Address correspondence and reprint requests to Steven A. Rosenberg, Surgery Branch, National Cancer Institute, National Institutes of Health, Building 10, Room 2B42; 10 Center Drive, Bethesda, MD 20892-1502; e-mail: sar@nih.gov

melanoma. Many of these vaccines have employed human leukocyte antigen (HLA) class I-restricted peptides, and although they can generate high levels of $CD8^+$ T cells capable of recognizing melanoma cells, clinical responses have been unpredictable and remain an elusive goal.[6]

In a pilot study[6] in which patients with metastatic melanoma were vaccinated with either the native class I-restricted gp100:209-217 peptide or a peptide with a methionine substitution for threonine at the second position, gp100:209-217(210M) (IMDQVPFSV), the modified peptide was superior to the native peptide in eliciting immune reactivity (91% versus 25% of patients, $P = 0.006$). A modified epitope for the MART-1 peptide in which lysine substitutes for the native alanine, MART-1:26-35(27L) (ELAGIGILTV), has also been noted in preliminary in vitro studies[7] to be more immunologic than the native MART-1:27-35 peptide.

A potential explanation for the paucity of in vivo clinical responses to these class I-restricted cancer peptide vaccinations is lack of appropriate "help" from $CD4^+$ T lymphocytes. $CD4^+$ T cells can regulate the growth, differentiation, and function of immunologic effector cells and thus may play a significant role in antitumor immunity[8,9] by elaborating cytokines and by inducing expression of adhesion and costimulatory molecules on the surface membrane of antigen-presenting cells.[10–13] Recognition of the importance of CD4 "help" has stimulated attempts to immunize cancer patients with class II-restricted epitopes derived from tumor antigens.

The class II allele HLA-DRB1*0401 is present in approximately 15% of patients with metastatic melanoma.[14] We have identified a DRB1*0401-restricted human gp100 epitope, gp100:44-59 (WNRQLYPEWTEAQRLD), using mice transgenic for chimeric human-murine DR4-IE.[15] $CD4^+$ T cells from these transgenic mice vaccinated with gp100:44-59 could recognize human tumors expressing gp100 and DRB1*0401 but not tumors lacking expression of either molecule. Human $CD4^+$ T cells sensitized with gp100:44-59 in vitro specifically recognized DRB1*0401-expressing Epstein-Barr virus-transformed B cells pulsed with this peptide.

We thus hypothesized that simultaneous immunization of melanoma patients with both class I- and class II-restricted melanoma peptides would generate both $CD8^+$ and $CD4^+$ T-cell activation and thus lead to improved clinical responses. In this publication, we report on the clinical and immunologic responses of two consecutive phase 2 trials: In the first protocol (Protocol 1), 22 patients with metastatic melanoma were vaccinated with two modified class I-restricted melanoma peptides, gp100:209-217(210M) and MART-1:26-35(27L). In the subsequent protocol (Protocol 2), 19 patients with metastatic melanoma were immunized with the same modified class I-restricted peptides, gp100:209-217(210M) and MART-1:26-35(27L), in conjunction with a class II-restricted peptide, gp100:44-59. Unexpectedly, as evaluated in peripheral blood lymphocytes, the addition of the class II peptide appeared to decrease immunity against the class I peptides.

PATIENTS AND METHODS

Patients

For both protocols, HLA-A*0201$^+$ patients with evaluable metastatic melanoma, no previous exogenous exposure to gp100 or MART-1, and an expected survival of greater than 3 months were eligible. All patients in Protocol 2 were also HLA-DRB1*0401$^+$. Exclusion criteria were serum creatinine greater than 2.0 mg/dL, total bilirubin greater than 2.0 mg/dL, alanine aminotransferase and aspartate aminotransferase greater than three times the upper limit of normal, white blood cell count less than 3000/mm^3, platelet count less than 90,000/mm^3, current pregnant or lactating status, Eastern Cooperative Oncology Group performance status greater than 2, the presence of any active systemic infection, symptomatic cardiac disease, autoimmune or immunodeficiency disease, and positivity for hepatitis BsAg or human immunodeficiency virus antibody. At least 3 weeks were required between enrollment into this protocol and any previous systemic therapy.

All patients were enrolled in these sequential trials between April 1999 and August 2000, and all signed an informed consent prior to protocol enrollment. Both protocols were approved by the Institutional Review Board of the National Cancer Institute. All patients were treated in the Surgery Branch at the Warren G. Magnuson Clinical Center of the National Institutes of Health in Bethesda, Maryland.

Vaccine Therapy

In Protocol 1, patients received 1 mg of gp100:209-217(210M) peptide emulsified in incomplete Freund adjuvant (IFA) injected subcutaneously in one extremity and 1 mg of MART-1:26-35(27L) peptide emulsified in IFA in another extremity every 3 weeks.

In Protocol 2, patients received the same peptides in IFA that were administered in the same fashion every 3 weeks as in Protocol 1, but each emulsion also contained 5 mg of the class II-restricted peptide gp100:44-59. All

peptides were prepared under Good Manufacturing Practice by Multiple Peptide Systems (San Diego, CA).

All patients underwent apheresis before treatment and 3 weeks after every two vaccinations to obtain peripheral blood lymphocytes for in vitro immunologic monitoring. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (ICN, Aurora, OH) separation and were cryopreserved at 10^8 cells/vial in heat-inactivated human AB serum with 10% dimethyl sulfoxide and stored at −180°C until further use.

Each course of treatment consisted of four cycles. Patients who completed one course of treatment and experienced either a minor, mixed, partial or complete response (as defined below) or stable disease received a second course. Patients who had progressive disease after receiving vaccines alone had the option of receiving IL-2 therapy.

IL-2 Therapy

Patients who opted for IL-2 therapy after disease progression with vaccines alone, and who had no contraindications to IL-2 administration, were given high-dose IV IL-2. For each cycle of treatment, IL-2 was started the day after vaccination and was administered as described in previous publications.[5,16] Briefly, 720,000 IU/kg of recombinant IL-2 (provided by Cetus Oncology Division, Chiron Corp., Emeryville, CA) was reconstituted from lyophilized powder in 5% human serum albumin and given as a 15-minute IV infusion for each dose. IL-2 was administered every 8 hours as tolerated for up to a maximum of 12 doses or until the development of a grade III or IV toxicity not easily reversed by supportive therapy, any evidence of neurologic toxicity, or patient refusal.

Clinical Response Evaluation

All patients underwent magnetic resonance imaging of the brain and computed axial tomography of the chest, abdomen, and pelvis within 4 weeks before starting treatment and subsequently after every two cycles of therapy. Radionuclide bone scans were also used for pretreatment staging: If they were positive for bony metastases, they were repeated at each evaluation point; if they were negative, they were not repeated again unless suggested by clinical symptoms. Photographs, plain radiographs, or other radiologic modalities were also used as needed to evaluate disease sites.

For every patient, the product of the maximum perpendicular diameters of all tumors before and after treatment was calculated. A partial response was defined as the reduction of ≥50% (but <100%) in the sum of the products of the maximum perpendicular diameters of all evaluable metastases lasting at least 1 month with no new or enlarging tumors; a minor response was the reduction of ≥25% but <50%; a complete response was the disappearance of all evaluable tumor sites for at least 1 month. A mixed response was defined as regression of some tumors but growth in others. Patients not achieving these criteria were deemed as having progressive disease. For final analysis, patients not having either a partial or complete response were deemed nonresponders.

In Vitro Sensitization Assay

In vitro sensitization (IVS) assay was used to assess immunologic reactivity as previously described.[6] Cryopreserved PBMC were thawed into complete media (CM: Iscove's Modified Dulbecco Media with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin), plated at 1.5×10^6 cells/mL with 1 μmol/L of native gp100:209-217 or MART-1:27-35 peptide and incubated overnight at 37°C and 5% CO_2. IL-2 was added on the following day for a final concentration of 300 IU/mL. Cells were split (and fresh media with 300 IU/mL IL-2 added) as needed and were harvested 11 to 13 days after initiation of the culture. Then 10^5 of these cells were co-incubated with 10^5 peptide-pulsed T2 cells in 200 μL CM per well in 96-well plates. The T2 cells were pulsed by incubating T2 cells at 2×10^6 cells/mL with 1 μmol/L peptide for 2 to 3 hours at 37°C and 5% CO_2. Alternatively, 10^5 melanoma cells were also used as stimulators. After 18 to 24 hours of co-incubation, interferon-γ (IFN-γ) release in the supernatant was measured using standard enzyme-linked immunosorbent assays (ELISA) (Pierce-Endogen, Rockford, IL).

The stimulation index (SI) was calculated using the ratio of IFN-γ released by PBMC due to co-incubation with T2 cells pulsed with the stimulating peptide compared with an irrelevant control gp100:280-288(288V) peptide. IVS assays were performed on PBMC pretreatment, post-2-vaccinations and post-4-vaccinations; each assay was repeated at least once. A positive assay is defined as IFN-γ ≥ 100 pg/mL, ≥2 times greater when compared with a control peptide (ie, when SI ≥ 2) and ≥2 times greater than preimmunization samples.

Enzyme-Linked Immunosorbent Spot Assay

To quantify the frequency of cells reactive to class I-restricted peptides, cryopreserved PBMC were thawed in an enzyme-linked immunosorbent spot (ELISPOT) as-

TABLE 1. *Patient demographics*

Patient trait	Protocol 1	Protocol 2
Vaccine received	Class I only	Class I + II
Total no. of patients (%)	22 (100%)	19 (100%)
Gender		
Male	14 (64%)	15 (79%)
Female	8 (36%)	4 (21%)
Age (y)		
Average	52.1	50.6
Range	31–77	28–79
ECOG score		
0	21 (95%)	18 (95%)
1	1 (5%)	1 (5%)
prior therapy		
Surgery	22 (100%)	19 (100%)
Chemotherapy	5 (23%)	11 (58%)
Immunotherapy	15 (68%)	11 (58%)
Radiotherapy	4 (18%)	6 (32%)
Hormonal	2 (9%)	0 (0%)
Any 2 or more	17 (77%)	15 (79%)
Any 3 or more	8 (36%)	10 (53%)

Class I = gp100:209–217(210M) and MART = MART-1:26–35(27L); Class II = gp100:44–59; ECOG, Eastern Cooperative Oncology Group.

say media (EAM: RPMI 1640 with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 25 mmol/L HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin) at 2×10^6 cells/mL and rested overnight at 37°C and 5% CO_2. MultiScreen-HA plates (Millipore, Bedford, MA) were also incubated overnight at room temperature with 100 μL of anti-human IFN-γ antibody (BioSource, Camarillo, CA) at 10 μg/mL. The plates were washed and blocked with EAM the following day for 1 hour to prevent nonspecific binding. Irradiated C1R-A2 cells (at 10^6 cells/mL) were pulsed with either 5 μmol/L influenza peptide:58–66 or 1 μmol/L melanoma peptide for 2 to 3 hours at 37°C and 5% CO_2; native gp100:209–217 or MART-1:27–35 peptide was used to assess for immunization status while irrelevant gp100:280–288(288V) was used as control. Peptide-pulsed C1R-A2 cells (10^5) were subsequently co-incubated with 10^5 PBMC in 200 μL EAM in each preblocked IFN-γ antibody-coated well. After 24 hours of co-incubation at 37°C and 5% CO_2, the cells were lysed by washing the wells with phosphate buffered saline (PBS) with 0.05% Tween-20. Biotinylated anti-IFN-γ antibody, 100 μL (BD Pharmingen, San Diego, CA), diluted to 2 μg/mL in PBS with 1% bovine serum albumin and 0.05% Tween-20, was added to each well and incubated at 4°C overnight. The plates were washed and developed the following day with avidin–alkaline phosphatase (Invitrogen, Carlsbad, CA) and then stained with BCIP/NBT Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Dots per well were scanned and computed in a "blinded" manner using an ImmunoSpot reader (Cellular Technology Ltd, Cleveland, OH).

The number of ELISPOTs per experiment were averaged over quadruplicate wells and were corrected by subtracting the background ELISPOTs due to PBMC co-incubation with unpulsed C1R-A2 cells. The corrected number of ELISPOTs (N: the number of peptide-reactive $CD8^+$ T cells) was then used to calculate the frequency of T-cell precursors by dividing into 10^5 (PBMC used per well): one precursor per (10^5/N).

To measure class II-reactive T-cell precursors, the ELISPOT assay was performed as above except that 50 μmol/L gp100:44–59 peptide was used directly to stimulate 10^5 PBMC in 200 μL EAM per well. A T-cell clone with known reactivity against the gp100:44-59 vaccine was used as a positive control.

RESULTS

Patients and Clinical Response

Patient characteristics are shown in Table 1 and were similar in both protocols, although more patients with a prior history of chemotherapy were enrolled in Protocol 2. No patient in Protocol 1 had an objective response to vaccine therapy alone (Table 2). One patient in Protocol 2 with a 6-year history of multiple resections of metastases to the brain, lymph nodes, and subcutaneous tissue entered the protocol with a 1-cm biopsy-proven inguinal node metastasis (as his only measurable disease) that disappeared gradually over two courses of vaccination, associated with the development of vitiligo. He remained without disease for a total of 18 months until he developed an adrenal mass and recurrence of the inguinal node metastasis.

Eleven nonresponding patients in Protocol 1 subsequently received IL-2 therapy, and two had objective clinical responses (18%), comparable with our previous experience with IL-2 alone. Of the 19 nonresponders to vaccines alone in Protocol 2, 11 were subsequently treated with IL-2, and none responded.

TABLE 2. *Clinical response*

Protocol	Total no. of patients	NR	PR	CR	PR + CR
1: Class I only	22	22	0	0	0
Moved to IL-2	11	9	2	0	2 (18%)
2: Class I + II	19	18	0	1	1 (5%)
Moved to IL-2	11	11	0	0	0

Class I = gp100:209–217(210M) and MART-1:26–35(27L); Class II = gp100:44–59; NR, no response; PR, partial response; CR, complete response.

Immunologic Response

Of 19 patients in Protocol 1 with PBMC available for testing using the IVS assay, 18 patients (95%) developed new anti-gp100:209-217 reactivity (Fig. 1) after two vaccinations while one patient already had pretreatment reactivity. Eight (44%) of 18 patients had pretreatment reactivity against the MART-1:27-35 peptide, and little change was seen after two or four vaccinations (Fig. 2).

Of 16 patients in Protocol 2 with PBMC available for IVS assays, eight patients (50%) developed new anti-gp100:209–217 reactivity (data not shown). In 10 patients who received four vaccinations with both class I- and II-restricted peptides, seven patients (70%) were reactive against gp100:209–217. As in Protocol 1, little new anti-MART-1:27–35 reactivity was generated as a consequence of vaccination in Protocol 2.

The decreased immunization rate against gp100:209-217 found in patients receiving both class I- and II-restricted peptides in Protocol 2 was surprising. To examine this phenomenon further, we directly compared the 16 patients in Protocol 2 with the 19 patients on Protocol 1 with PBMC available. To ensure that any

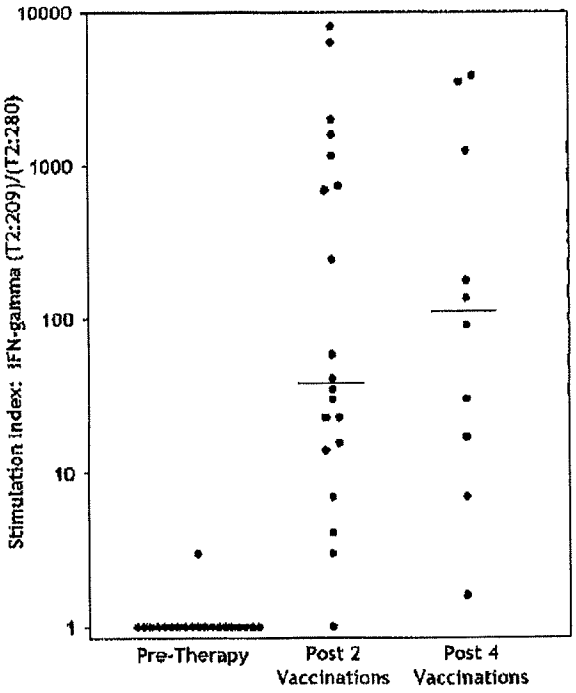


FIGURE 1. Reactivity against gp100:209–217 using PBMC of patients receiving only class I-restricted peptides using IVS assays. IFN-γ release was measured after PBMC co-incubation with T2 pulsed with 1 μmol/L of either gp100:209–217 or irrelevant gp100:280–288(288V). The horizontal line shows the median value.

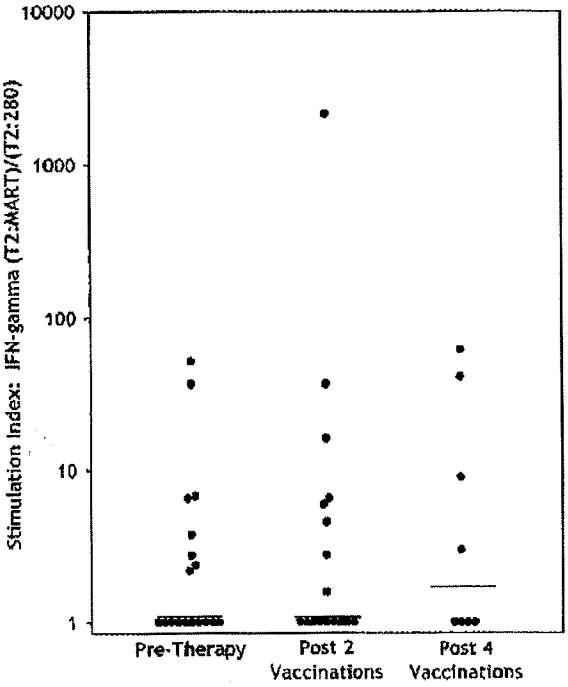


FIGURE 2. Reactivity against MART-1:27-35 using PBMC of patients receiving only class I-restricted peptides using IVS assays. IFN-γ release was measured after PBMC co-incubation with T2 pulsed with 1 μmol/L of either MART-1:27-35 or irrelevant gp100:280-288(288V). The horizontal line shows the median value.

differences in the IVS assay results were not due to reagent differences, the assays on patients in both protocols were repeated and performed at the same time using cryopreserved samples. As seen in Figure 3, the SI of patients receiving both class I- and class II-restricted peptides was significantly decreased compared with the SI of those receiving only class I-restricted peptides ($P = 0.01$ by Mann-Whitney U test).

The level of immunization in both protocols was at the limit of detection of the ELISPOT assay, and the majority of patients generated fewer than 10 ELISPOTs per 10^5 cells. As seen in Table 3, using PBMC obtained after four vaccinations, a slight trend toward decreased frequency of gp100:209–210-reactive $CD8^+$ T-cell precursors was noted in the group receiving both class I- and II-restricted peptides ($P = 0.16$ by Mann-Whitney U test); however, the number of ELISPOTs per 10^5 PMBC was too small to draw reliable conclusions. Results from the ELISPOT assay evaluating reactivity against the MART-1:27-35 peptide were also below the limit of detection (data not shown).

To assess whether the decreased reactivity toward gp100:209-217 seen in patients receiving both the class I- and II-restricted peptides was due to physical factors

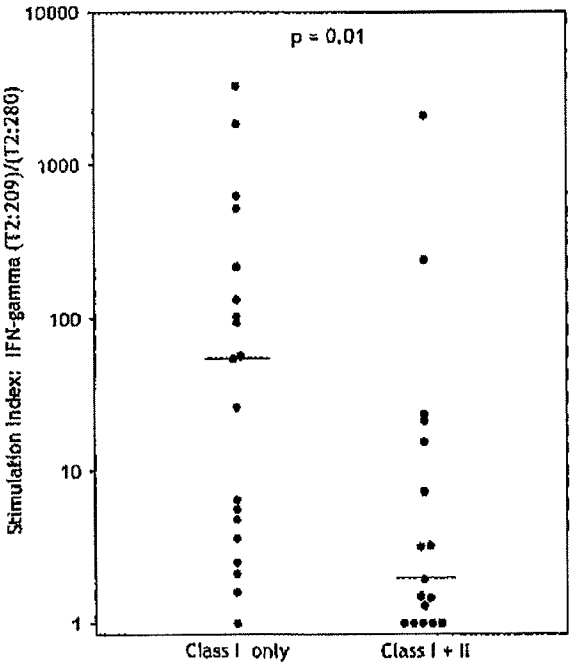


FIGURE 3. Comparison of the reactivity against gp100:209–217 between patients receiving only class I-restricted peptides and those receiving both class I- and II-restricted peptides. IFN-γ release was measured after PBMC co-incubation with T2 pulsed with 1 μmol/L of either gp100:209–217 or irrelevant gp100:280–288(288V). The horizontal line shows the median value: $P = 0.01$ by Mann-Whitney U test.

from having the two peptides mixed prior to vaccination, "mixing studies" were performed as follows: In one experiment (Table 4), a T-cell clone with known anti-gp100:209–217(210M) reactivity was incubated overnight with serial dilutions of either 1) 1 μmol/L gp100:209–217(210M), 2) 1 μmol/L gp100:209–217(210M) mixed with 5 μmol/L gp100:44–59 or 3) 5 μmol/L gp100:44–59. IFN-γ release measured using ELISA found no evidence of inhibition of gp100:209–217(210M)-reactive $CD8^+$ T cells by in vitro co-incubation with the mixture of gp100:44–59 and gp100:209–217(210M). In another "mixing" experiment, the IVS assay was performed on reactive PBMC (of several patients from Protocol 1) that were stimulated with either 1 μmol/L gp100:209–217(210M) alone or 1 μmol/L gp100:209–217(210M) mixed with 5 μmol/L gp100:44–59. No differences were seen between PBMC stimulated with only gp100:209–217(210M) or with gp100:209–217(210M) mixed with gp100:44–59 (data not shown).

Both IVS and ELISPOT assays failed to demonstrate any reactivity against the gp100:44–59 peptide in either pretreatment or post-4-vaccinations PBMC (data not shown).

DISCUSSION

Immunization with HLA class I-restricted tumor peptides in cancer patients can result in the generation of circulating $CD8^+$ cytotoxic T lymphocytes (CTL) with antitumor reactivity, although tumor regression in these patients is uncommon. $CD8^+$ cells, although crucial, are only part of the complex ensemble of participants involved in mediating tumor regression. $CD4^+$ T cells have been shown to be essential in providing "help" to $CD8^+$ cells.[8–13] Activation of $CD4^+$ cells by recognition of the appropriate epitopes presented by the HLA class II molecules on antigen presenting cells (APC) causes the expression of CD40L, which engages CD40 on APC, in turn "conditioning" these APC to express costimulatory molecules (such as B7-1 and B7-2). These activated APC subsequently can further activate $CD8^+$ T cells not only through the HLA class I-epitope-T-cell receptor pathway, but also through B7-CD28 costimulation. Furthermore, activated $CD4^+$ helper cells also release cytokines

TABLE 3. *$CD8^+$ T cell precursors reactive against gp100:209–217*

Patients	N = corrected ELISPOTs per 10^5 PBMC	1 Precursor per (10^5/N)
Class I only	Median = 4.6*	
1	4.8	20,833
2	4.1	24,390
3	0.3	333,333
4	0	—
5	5.1	19,608
6	4.4	22,727
7	2.9	34,483
8	12.9	7,752
9	8.3	12,048
10	4.3	23,256
Class I + II	Median = 1.5*	
1	0	—
2	1.2	83,333
3	2.8	35,714
4	0.4	250,000
5	1.8	55,556
6	178.3	561
7	8.2	12,195
8	0.1	1,000,000
9	0.5	200,000
10	2.3	43,478

* $P = 0.16$ by Mann-Whitney U test. Class I = gp100:209–217(210M) and MART-1:26–35(27L); Class II = gp100:44–59; corrected = the background number of ELISPOTs due to PBMC co-incubation with unpulsed CIR-A2 cells were subtracted from the number of ELISPOTs due to co-incubation with gp100:209–217-pulsed CIR-A2.

TABLE 4. *IFN-γ (pg/mL) released by anti-gp100:209–217(210M) T-cell clone with serial dilutions of gp100:209–217(210M) ± gp100:44–59*

Dilution of peptides	gp100:209–217(210M) alone		gp100:209–217(210M) + gp100:44–59		gp100:44–59 alone	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
1	2289	2428	2624	2466	1	0
1:100	1532	2022	2182	1851	1	0
1:500	913	1123	1205	1132	5	1
1:2500	512	670	702	600	0	0
1:12,500	45	83	255	96	5	0
1:62,500	0	2	61	4	0	2
1:31,250	0	0	8	0	0	2

Dilution of "1" = 1 μmol/L gp100:209–217(210M); 1 μmol/L gp100:209–217(210M) mixed with 5 μmol/L gp100:44–59; or 5 μmol/L gp100:44–59. Rep, replicate.

(such as IL-2) important in CTL proliferation and differentiation and in recruitment of other effector cells, such as eosinophils and macrophages, which may be important in mediating antitumor reactivity.[8,9]

In an effort to evaluate "help" from $CD4^+$ cells, we vaccinated melanoma patients using either class I-restricted epitopes alone or both class I- and class II-restricted epitopes from melanoma-associated antigens. Analysis of PBMC from patients in the two sequential protocols presented here suggested that the successful immunization against a class I-restricted gp100 melanoma peptide, as assessed in circulating lymphocytes, was unexpectedly decreased by simultaneous immunization with a class II-restricted gp100 peptide. Only 50% of patients (8 of 16) receiving both class I- and II-restricted peptides showed evidence of new immunization against gp100:209–217 after two vaccinations, in contrast to 95% of patients (18 of 19) receiving only class I-restricted peptides ($P_2 < 0.005$; Fisher's exact test). A pilot trial evaluating the same gp100:209–217(210M) peptide had shown previously that 91% of patients (10 of 11) receiving only this peptide were successfully immunized after two vaccinations.[6] Although there were two outliers in the group immunized with both class I- and II-restricted epitopes who had high SI values (241 and 2112; Fig. 3) against gp100:209–217, these patients were among the clinical nonresponders. With the ELISPOT data, there was a hint that patients vaccinated with both class I- and II-restricted peptides had fewer class I-reactive $CD8^+$ T-cell precursors, but overall values were too close to the limits of detection by this assay for accurate assessment. With the MART-1:26-35(27L) peptide, we were not successful in vaccinating patients on either protocol.

What are possible explanations for the seemingly subdued clinical and immunologic responses in patients immunized with both class I- and II-restricted epitopes? The sequential, nonrandomized conduct of the two trials could have resulted in an undetected referral and selection bias in the enrollment of specific patients to the protocols. In particular, since the patients in Protocol 2 were required to be HLA-DRB1*0401$^+$, the decreased anti-gp100:209–217 reactivity seen in these patients might be due to intrinsically decreased responsiveness of people with this genotype. Perhaps the prior history of chemotherapy (which was seen in more patients on Protocol 2) negatively affected vaccine responsiveness, although all patients were required to be at least 3 weeks from any prior therapy and had normal cell counts and differential prior to protocol enrollment. The decrease in class I-restricted reactivity when the gp100:44-59 class II-restricted peptide was added does not appear to be due to direct physical interaction between the peptides (Table 4). Increasing evidence has shown the importance of $CD4^+CD25^+$ T cells with regulatory capabilities[17–21] that are naturally suppressive and antigen-nonspecific in their effector functions.[22] Recent studies have also shown that engagement of cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) on these $CD4^+CD25^+$ T cells by APC activates these cells,[23] leading to increased suppressive activities. Woo et al.[24] have shown that a high percentage of tumor-infiltrating lymphocytes from lung cancer specimens were $CD4^+CD25^+$ T cells; moreover, 80% of these $CD4^+CD25^+$ T cells expressed CTLA-4 and suppressed proliferation of autologous PBMC. It is possible that the unintended immunization and activation of these $CD4^+CD25^+$ regulatory T cells may have inhibited the development of class I-restricted reactivity. In pilot studies, no differences were seen in the levels of $CD4^+CD25^+$ T cells between pre- and posttreatment cryopreserved PBMC samples in our patients on Protocol 2 (data not shown); however, it is possible that the majority of these regulatory T cells are located at sites of inflammation, such as tumor sites, and not in the peripheral blood, thus

limiting our ability to identify and enumerate them. It is also possible that immunization with the gp100:44–59 peptide affected $CD8^+$ cell survival by triggering activation-induced cell death, mediated possibly through cytokines or Fas/FasL. On the other hand, we were not able to demonstrate, using IVS and ELISPOT assays, that vaccination with the class II-restricted gp100:44–59 elicited a $CD4^+$ response against the gp100:44–59 peptide. Furthermore, the assessment of immune reactivity in patients receiving peptide immunization reported here was conducted using peripheral circulating lymphocytes, and thus these results may not reflect immune reactions at the tumor site. Further studies of lymphocytes infiltrating into tumors following immunization are required.

This study has not detected a beneficial impact of adding immunization with a class II-restricted peptide to simultaneous immunization with a class I-restricted peptide. Indeed, a negative impact may exist with the addition of the class II-restricted peptide. Randomized trials will be required to answer this question.

Acknowledgment: The authors thank the Immunotherapy Fellows in the Surgery Branch and the clinical nursing staff of the 2-East and 2-J Units of the Magnuson Clinical Center of the National Institutes of Health for excellent patient care and support, and Susan Strobl and Kimberly Shafer-Weaver of the NCI-Frederick Cancer Research and Development Center for their invaluable assistance with the ELISPOT assays.

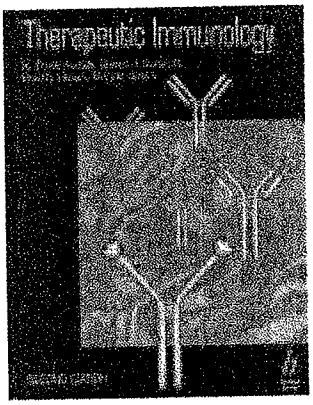
REFERENCES

1. Lotze MT, Dallal RM, Kirkwood JM, et al. Cutaneous melanoma. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer: Principles and Practice of Oncology*, 6th ed. Philadelphia: Lippincott Williams and Wilkins, 2001; pp 2012–2069.
2. Rosenberg SA, Yang JC, White DE, et al. Durability of complete responses in patients with metastatic cancer treated with high-dose interleukin-2. *Ann Surg.* 1998;228:307–319.
3. Atkins MB, Lotze MT, Dutcher JP, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol.* 1999;17:2105–2116.
4. Atkins MB, Kunkel L, Sznol M, et al. High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update. *Cancer J Sci Am.* 2000;6(suppl 1):S11–S14.
5. Phan GQ, Attia P, Steinberg SM, et al. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. *J Clin Oncol.* 2001;19:3477–3482.
6. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med.* 1998; 4:321–327.
7. Valmori D, Fonteneau JF, Lizana CM, et al. Enhanced generation of specific tumor-reactive CTL in vitro by selected Melan-A/MART-1 immunodominant peptide analogues. *J Immunol.* 1998;160:1750–1758.
8. Pardoll DM, Topalian SL. The role of $CD4^+$ T cell responses in antitumor immunity. *Curr Opin Immunol.* 1998;10:588–594.
9. Hung K, Hayashi R, Lafond-Walker A, et al. The central role of $CD4^+$ T cells in the antitumor immune response. *J Exp Med.* 1998; 188:2357–2368.
10. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature.* 1998;393:474–478.
11. Bennett SRM, Carbone FR, Karamalis F, et al. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature.* 1998; 393:478–480.
12. Schoenberger SP, Toes RE, van der Voort EI, et al. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature.* 1998;393:480–483.
13. Grewal IS, Flavell RA. A central role of CD40 ligand in the regulation of $CD4^+$ T-cell responses. *Immunol Today.* 1996;17:410–414.
14. Marincola FM, Shamamian P, Rivoltini L, et al. HLA associations in the antitumor response against malignant melanoma. *J Immunother Emphasis Tumor Immunol.* 1995;18:242–252.
15. Touloukian CE, Leitner WW, Topalian SL, et al. Identification of a MHC class II-restricted human gp100 epitope using DR4-IE transgenic mice. *J Immunol.* 2000;164:3535–3542.
16. Rosenberg SA, Yang JC, Topalian SL, et al. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA.* 1994;271:907–913.
17. Sakaguchi S, Sakaguchi N, Shimizu J, et al. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplant tolerance. *Immunol Rev.* 2001;182:18–32.
18. Stephens LA, Mottet C, Mason D, et al. Human $CD4^+$ $CD25^+$ thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol.* 2001;31:1247–1254.
19. Suri-Payer E, Amar AZ, Thorton AM, et al. $CD4^+$ $CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol.* 1998;160:1212–1218.
20. Thorton AM, Shevach EM. $CD4^+$ $CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med.* 1998;188:287–296.
21. Javia LR, Rosenberg SA. $CD4^+CD25^+$ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens. *J Immunother.* 2003;26:85–93.
22. Thorton AM, Shevach EM. Suppressor effector functions of $CD4^+$ $CD25^+$ immunoregulatory T cells is antigen nonspecific. *J Immunol.* 2000;164:183–190.
23. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+$ $CD4^+$ regulatory cells that control intestinal inflammation. *J Exp Med.* 2000;192:295–302.
24. Woo EY, Yeh H, Chu CS, et al. Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol.* 2002;168:4272–4276.

Therapeutic Immunology · SECOND EDITION

K. Frank Austen, Steven J. Burakoff, Fred S. Rosen, Terry B. Strom

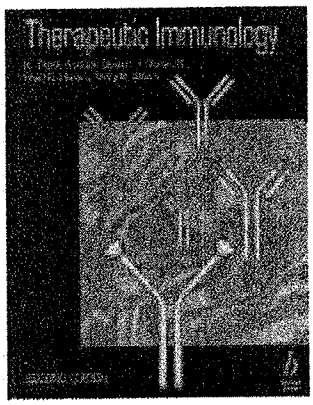
Therapeutic Immunology
著者: Karl Frank Austen, Steven J. Burakoff, Fred S. Rosen, Terry B. Strom
寄与者 Karl Frank Austen
出版社: Blackwell Publishing, 2001
ISBN 0632043598, 9780632043590



Therapeutic Immunology · SECOND EDITION

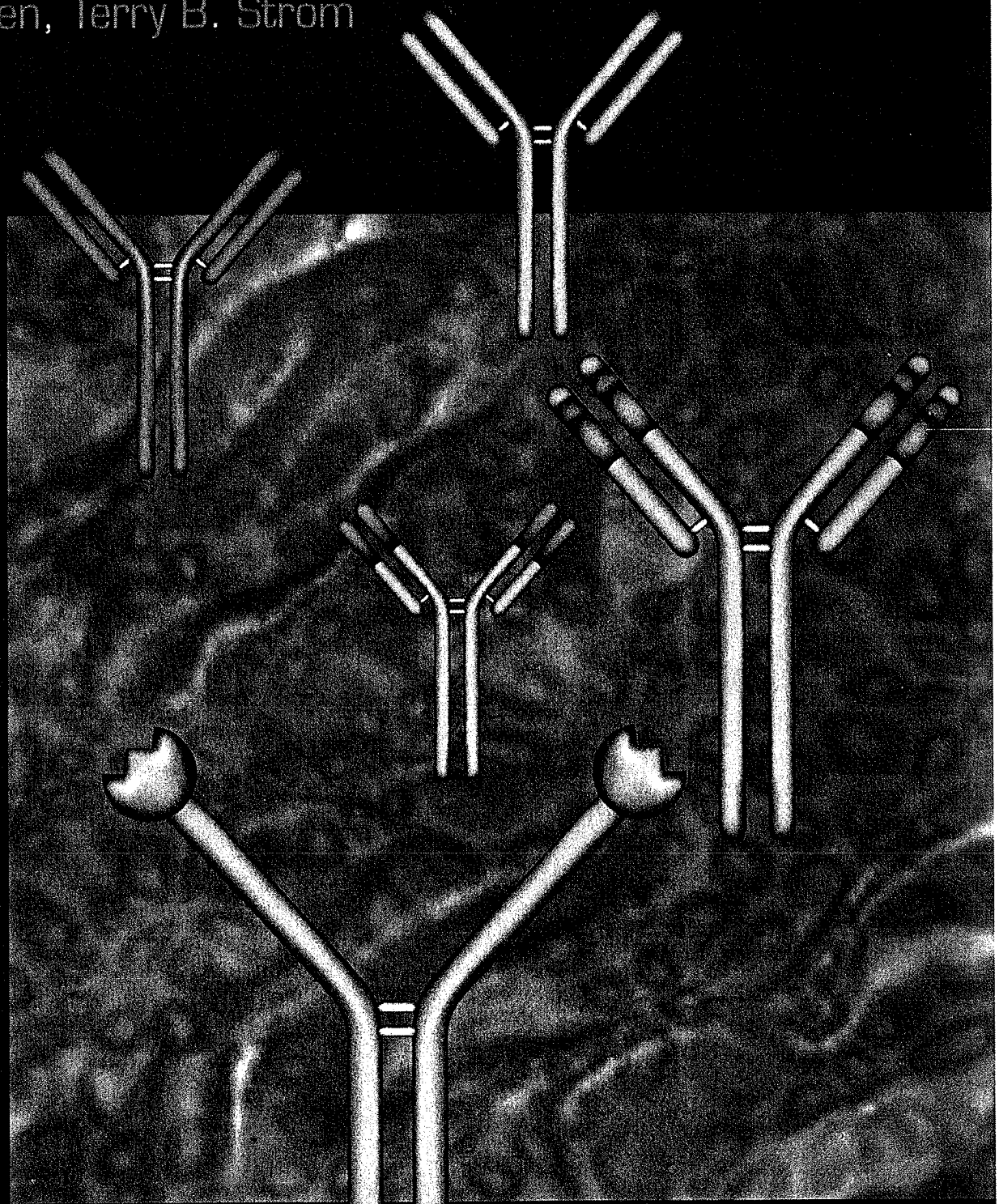
K. Frank Austen, Steven J. Burakoff, Fred S. Rosen, Terry B. Strom

Therapeutic Immunology
著者: Karl Frank Austen, Steven J. Burakoff, Fred S. Rosen, Terry B. Strom
寄与者 Karl Frank Austen
出版社: Blackwell Publishing, 2001
ISBN 0632043598, 9780632043590



Therapeutic Immunology

K. Frank Austen, Steven J. Burakoff,
Fred S. Rosen, Terry B. Strom



SECOND EDITION

b

Therapeutic Immunology

Second Edition

Edited by

K. Frank Austen, MD

Theodore Bevier Bayles Professor of Medicine
Harvard Medical School
Director, Inflammation & Allergic Diseases Research Section
Division of Rheumatology, Immunology, and Allergy
Department of Medicine
Brigham and Women's Hospital
Boston, Massachusetts

Steven J. Burakoff, MD

Laura and Issac Perlmutter Professor of Pathology
Professor of Medicine and Pediatrics
New York University School of Medicine
Director of the New York University Kaplan Comprehensive Cancer Center
Director of the Skirball Institute for Biomolecular Medicine
New York, New York

Fred S. Rosen, MD

James L. Gamble Professor of Pediatrics
Harvard Medical School
President
The Center for Blood Research
Boston, Massachusetts

Terry B. Strom, MD

Professor of Medicine
Harvard Medical School
Director of Immunology
Beth Israel Deaconess Medical Center
Boston, Massachusetts

b

Blackwell
Science

Editorial Offices:
Commerce Place, 350 Main Street, Malden, Massachusetts 02148, USA
Osney Mead, Oxford OX2 0EL, England
25 John Street, London WC1N 2BL, England
23 Ainslie Place, Edinburgh EH3 6AJ, Scotland
54 University Street, Carlton, Victoria 3053, Australia
Other Editorial Offices:
Blackwell Wissenschafts-Verlag GmbH, Kurfürstendamm 57, 10707 Berlin, Germany
Blackwell Science KK, MG Kodenmacho Building, 7-10 Kodenmacho Nihombashi, Chuo-ku, Tokyo 104, Japan

Acquisitions: Chris Davis
Development: Julia Casson
Production: Andover Publishing Services
Manufacturing: Lisa Flanagan
Marketing Manager: Anne Stone
Director of Marketing: Lisa Larsen
Cover design by Electronic Illustrators Group
Typeset by Best-set Typesetter Ltd., Hong Kong
Printed and bound by Edwards Brothers/Ann Arbor

Printed in the United States of America
00 01 02 03 5 4 3 2 1

Distributors:
USA
Blackwell Science, Inc.
Commerce Place
350 Main Street
Malden, Massachusetts 02148
(Telephone orders: 800-215-1000 or 781-388-8250;
fax orders: 781-388-8270)

Canada
Login Brothers Book Company
324 Saulteaux Crescent
Winnipeg, Manitoba, R3J 3T2
(Telephone orders: 204-837-2987)

Australia
Blackwell Science Pty, Ltd.
54 University Street
Carlton, Victoria 3053
(Telephone orders: 03-9347-0300;
fax orders: 03-9349-3016)

Outside North America and Australia
Blackwell Science, Ltd.
c/o Marston Book Services, Ltd.
P.O. Box 269
Abingdon
Oxon OX14 4YN
England
(Telephone orders: 44-01235-465500;
fax orders: 44-01235-465555)

Library of Congress Cataloging-in-Publication Data

Therapeutic immunology / by K. Frank Austen . . . [et al.].—2nd ed.
p.; cm.
Includes bibliographical references and index.
ISBN 0-632-04359-8
1. Immunotherapy. I. Austen, K. Frank (Karl Frank)
[DNLM: 1. Immunotherapy. 2. Immune System—drug effects. QW 940 T398 2001]
RM275 .T44 2001
615¢37—dc21

00-044408

Contents

Table 1-1. Comparison of the Responses to Carbohydrates and Proteins

	Pure Polysaccharide	Globular Protein
Recognition by BCR	Yes	Yes
Recognition by TCR	No	Yes, after processing
Binding to MHC	No	Only after processing by APC
Involvement of CD4 T cells	No	Yes
Memory	Poor	Strong
Affinity maturation	Poor	Strong

domain. In contrast to the BCR, the TCR does not engage proteins directly, but only recognizes peptides or unfolded proteins complexed to histocompatibility molecules (see below).

There is, of course, much diversity in the recognition properties of the receptors. Presumably, the number of entities that the receptors can recognize reaches 10^{10}. This ensures that most chemical conformations can eventually be identified by the lymphocyte and that most pathogens can be recognized. The molecular explanation for diversity was first solved at the laboratory of Susumu Tonegawa when various DNA segments were found to encode for the mature light chain of the BCR (28). The same findings were later made regarding the TCR. The gene that encodes the mature receptor is generated by recombining distinct gene segments found in the germ line (28–31). This selection of DNA segments from a finite number of V (for variable), J, and, for some chains, D gene segments plus addition or deletion of nucleotides at their coding ends (N-region diversity) is responsible for the generation of the very diverse library of B- and T-cell receptors. (For the Ig genes, the heavy chain is encoded by V, D, and J genes, while the light chain is encoded by V and J genes. The mature T-cell receptor alpha chain is encoded by recombination of V and J, while the beta chain encodes for V, D, and J gene segments. The VDJ exon is then spliced to genes encoding the constant segments of the heavy or light chain of Ig, or the TCR chains.) During their differentiation, the immature B and T cells express genes encoding two enzymes involved in the recombination process (these are the recombination-activating genes, RAG-1 and RAG-2) (32). These proteins are responsible for DNA scission and their joining (together with other proteins such as DNA ligase, Ku70, and Ku80). The RAG proteins are expressed at particular stages in the differentiation of B and T cells, a point that we discuss later.

Does the specificity of the antigen receptors change during an immune response? In the B cell, the quality of antigen recognition improves markedly as the response "matures." Affinity maturation was discovered by studying the response to haptens and finding by equilibrium dialysis that the affinity increased by several fold (33). At the molecular level, the change in affinity is explained by the presence of somatic mutations. During the B-cell response in germinal centers, the rate of mutation of the genes encoding the BCR increases markedly. This hypermutation was first indicated by Weigert, Cohn, and associates (34) when studying lambda light chains in the mouse. It results in point mutations in the variable genes, while the genes encoding the constant region are not affected. Somatic mutation takes place in the germinal centers of the follicles and is accompanied by extensive proliferation. It requires interaction of the B cells with CD4 T cells. Indeed, the response to pure polysaccharides that trigger B cells to a limited amount and do not stimulate T cells shows limited if any affinity changes. (For a comparison of the responses to proteins and polysaccharides, see Table 1-1.) Many of the growing B cells die, while those having the highest-affinity receptors survive and become the predominant clones. Thus, as a combination of somatic mutation and selection, the B-cell response improves for the best. These changes in affinity are not found in the T-cell response.

Antigen Presentation

The modes of interaction with antigen of the two major lineages of cells are different. B cells can directly recognize antigen molecules through their BCR: they can recognize pure carbohydrate as well as proteins in their native or tertiary configuration (Table 1-1). It is apparent that this direct recognition was the case when the specificity of serum antibodies was studied: antibodies raised against globular proteins bind only to the native molecule and not to the denatured proteins (reviewed in 1). Thus, most B-cell clones have been selected by protein antigens prior to their extensive catabolism by the host. This is not the case for T cells, where an antigen-presenting cell (APC) has to come into operation in order to promote the recognition. T cells recognize a linear sequence of amino acids, either segments of an unfolded protein or peptides of a few amino acids. Indeed, the TCR will not interact directly with protein antigens unless they are part of a complex with the major histocompatibility complex (MHC) molecules. Thus, protein antigens need to be processed and presented by the APC as peptides or unfolded molecules bound to MHC proteins (35). Pure polysaccharides do not bind to MHC proteins and do not stimulate T cells (Table 1-1).

The differences in antigen recognition between T and B cells was a major puzzle for many years. The early literature had indicated two sets of reactions to proteins. As mentioned above,

Table 1-2. Comparison of Class I and II MHC Molecules

	MHC-I	MHC-II
Expression	Most cells	Primarily in APC and in some specialized epithelial cells
Structure	A heavy transmembrane chain (~44 Kd) together with β2 microglobulin (12 Kd)	Two transmembrane chains α and β of ~30 Kd
Peptide binding	Usually 8–10 residues in length	Normally large, >5 residues
Peptide loading	In endoplasmic reticulum	In vesicles
Site of catabolism of the protein	Cytosol: proteasome	Vesicles
Chaperonins	Tapasin, calreticulin	Invariant chain, AL-A DM
Biology	Antiviral and antitumor immunity	Immunity to intracellular pathogenesis and foreign proteins
	Interacts with CD8 T cells	Interacts with CD4 T cells

serum antibodies reacted with conformational-dependent epitopes of a globular protein. The same results applied to immunopathologic reactions, such as the Arthus reaction or the acute anaphylactic reactions, both of which needed to be elicited with the native proteins. In contrast were the reactions now known to be elicited by T cells, such as the delayed sensitivity reactions. These reactions were identified when Koch first reported the reaction to tuberculin, and were later shown, by the classical experiments of Chase and Landsteiner, to be transferred by lymphocytes (36,37). These reactions did not discriminate between denatured and native proteins and showed marked dependency on the "carrier," in the examples of hapten-carrier protein conjugates. (The antibody-mediated reactions were infiltrated by neutrophils, while the delayed reactions were rich in activated macrophages, an indication of the different patterns of inflammation, which involved complement and cytokines, respectively.)

The seminal molecules responsible for the APC–T cell interaction are those encoded in the major histocompatibility gene complex (MHC) of the species. The APC informs the T cell of the molecular status of its environment by displaying peptides bound to either their class I or class II MHC molecules (Table 1-2). MHC molecules are peptide-binding proteins that rescue peptides from intracellular digestion (38). The peptide-MHC complex constitutes the epitope that the TCR recognizes. The TCR contacts both the peptide as well as residues of the MHC molecule, an issue that has now been settled by x-ray crystallographic analysis of TCR complexed to their specific peptide-MHC complex (39–41). This double interaction creates a situation whereby the T cell that is selected in the thymus must have two specificities, one for peptides and the other for its own MHC. This interaction results in the phenomenon of MHC restriction—that is to say that T cells recognize foreign elements, but always in the framework of also recognizing a self-MHC protein (42–45).

The MHC molecules were discovered in the context of transplantation reactions (46,47). Indeed, it is the strong reaction to allogeneic MHC proteins that is responsible for graft rejection, creating the difficulties in transplanting tissues within members of the species. These proteins were found to be part of a complex genetic system with a high degree of allelism. It is the most polymorphic gene system of the species.

The human leukocyte antigen (HLA) system was discovered when sera from mutiparous women or highly transfused individuals were found to contain leukoagglutinins—i.e., anti-HLA antibodies, which is the basis of tissue typing for transplantation reactions (48,49). However, it was through mouse genetics and the development of inbred strains of mice that the major advances in this field were made (50). These lines were developed through the laborious work of many, including CC Little and George Snell at the Jackson Laboratory. These strains provided a homogeneous genetic population that enabled the fine genetic dissection of the mouse MHC (the H-2) and cell transfers and organ grafts to be carried out (51). The basic laws of transplantations were developed through the use of inbred strains of mice. The physiological role of MHC was discovered in part through serendipity, but by very astute researchers who followed the leads and pinpointed the essential role of the MHC in T-cell recognition. Indeed, the realization that some strains of mice and guinea pigs were either high or low responders to peptides and that this response was linked genetically to the MHC laid the groundwork for such discoveries (52–55). Finally, considering this topic of inbred mice, the next major advance of the past decade has been the production of mice bearing transgenes or having mutations

or ablations of a given gene. The advances with the use of transgenes or gene knockout mice has been revolutionary, dramatically changing biology and immunology (56).

There are two sets of MHC molecules encoded in the MHC gene complex, the class I and class II molecules (57–60) (Table 1–2). Each MHC gene complex may contain more than one gene encoding class I or II molecules. In the human HLA there are three major class I proteins, HLA A, B, and C, and three major class II proteins, HLA DR, DQ, and DP. Each has multiple allelic variants. The class I molecules are made up of a heavy chain and a small polypeptide beta-2 microglobulin (61). The heavy chain is a transmembrane protein that contains three external domains; the two most distal to the membrane assemble to form the combining site while the one closest to the membrane is made of an Ig-like fold. The class II molecules are composed of two transmembrane chains, each having an external domain that contributes to the formation of the peptide combining site (62). The peptide combining site of both sets of molecules has a similar basic structure, made up of a platform of seven beta pleated sheet strands, bordered by two walls made of alpha helices, with the peptide sitting at about the center between the helices. The amino acids responsible for the allelic polymorphisms are all located in the helices and platform, in contact with the peptide. Thus, the driving force in evolution for diversification of the MHC is at the level of peptide binding.

MHC molecules do not discriminate between self-peptides and foreign peptides—both bind or do not bind (63). Indeed, these molecules are normally occupied by autologous peptides that bind with varying degrees of affinity (64–66). It is estimated that for each APC, about 2000 peptides are contained in each set of MHC molecules. The importance of these self-peptides is threefold. First, it allows for the elimination in the thymus of T cells that are autoreactive. This is the important process of "negative selection" or central tolerance, which is discussed below. Second, binding of such peptides gives stability and structure to the MHC molecule, which otherwise would not be correctly expressed: depending on the binding affinity, the MHC molecule will be able to exchange some of these self-peptides with other peptides, including those from foreign molecules. But third, and very importantly, the self-peptides bound to the MHC molecule are the basis for T-cell recognition in autoimmunities. There is a relationship between a particular autoimmunity and an MHC allele such as occurs in type I or insulin-dependent diabetes (67). At this point in time, the number of autologous peptides identified as responsible for an autoimmune process is very limited, but the approaches and technologies are now available, and information should be forthcoming.

Class I molecules interact with peptides of 8–10 residues in length, but of much larger length in the case of class II molecules (61,65,68,69). Peptides bind through two major sets of interactions. One set of interactions is at the level of amino acid side chains with sites (or pockets) in the combining site that are allele specific: in a class I molecule, there are two major sites located at each end. Four sites are involved in peptide binding to class II, although usually one becomes the dominant site of interaction. A second set of interactions involves a complex hydrogen bonding network between conserved residues of the MHC and the peptide backbone (for examples, see 70 and 71).

Peptides vary in their strength of binding to MHC, depending on their amino acid composition as well as on the particular MHC alleles (72). In general, binding is in the low nM to μM affinity range. Each allele binds to many peptides. So there is specificity, but at the same time the binding is broad. This binding repertoire allows an individual to recognize multiple peptides. This was well stated by Benacerraf: "The evolutionary significance of the commitment of T cells to MHC antigens should be assessed from several vantage points. From the point of view of the individual concerned, the existence of such a broadly polymorphic system to determine specific responsiveness and suppression will inescapably result in individuals with different immunological potential to a given challenge. Some will clearly be at risk, whereas others will be better prepared to resist certain infectious agents, and it is not surprising that immunological diseases are linked to the MHC. As far as the species is concerned, this polymorphic defense system results in a very significant survival advantage to unforeseen challenges and a better possibility for the immune system to adapt to evolutionary pressures" (55).

The probable reason for the development of two sets of MHC molecules is because each samples a distinct cellular compartment. Thus, the class I MHC proteins sample the peptides from the cytosol (73, reviewed in 74). These peptides result from proteasomal digestion, passing into the endoplasmic reticulum (ER) by way of peptide transporters of the ABC type (which also happen to be encoded in the HLA gene complex). In the ER, the complex assembles with nascent class I MHC molecules and is then transported to the plasma membrane (75). Several chaperonin-like molecules such as tapasin and calreticulin are involved, allowing for the proper folding of the heavy chain, the peptide, and beta-2 microglobulin. It follows that the MHC class I system is the one that evolved to deal with viral infections, since it is in the cytosol that viral proteins are assembled and processed. Of the two major subsets of T cells, the CD8 T cells recognize the peptide-MHC class I complex. The CD8 molecule has affinity for the class I MHC molecule and contributes to the cellular interaction. CD8 T cells protect against viral infections by killing infected cells and also by releasing interferon-gamma (reviewed in 76 and 77).

In contrast, the class II MHC system is best suited to handle peptides from proteins that have been taken into the APC by endocytosis (69). The internalized protein, either free or as part of a microbial structure, is processed in organelles of low pH and the protein is unfolded and subjected to partial proteolysis—the peptide or unfolded protein binds to nascent class II molecules in a complex series of steps that involve a number of auxiliary or chaperoning proteins (invariant chain, HLA-Dm). So it follows that the class II system is primarily involved in the reactions to exogenous proteins or to microbes that reside in phagolysosomes. Infections with intracellular bacteria depend on the action of the CD4 T-cell subset interacting with the microbial peptides selected by the class II MHC molecules. The CD4 molecule, as in the case of the CD8 molecule, contributes to the interaction while at the

same time serving to mark this subset. CD4 T cells are involved in activating macrophages by releasing interferon-gamma, promoting growth via IL-2, and regulating B-cell differentiation by way of IL-4 release (reviewed in 78).

The three cells involved in antigen presentation are the dendritic cell, which derives from monocytes, expresses a high level of MHC molecules, and is specially endowed for very effective close contact with lymphocytes (79,80); the monocyte/macrophage, which modulates its expression of MHC molecules and responds to interferon gamma by increasing the number of them; and the B cell, which expresses MHC molecules constitutively. These three cells are denoted as antigen-presenting cells, or APCs. Antigen presentation in a lymph node takes place in two main contexts. First is the activation of the CD4 T-cell clones when antigen enters the node and is captured by the dense network of dendritic cells in the deep cortex. The dendritic cells are powerful APC with a high density of MHC molecules and a large surface made by cytoplasmic extensions that favor close cellular interactions (79). The second context is that of B cell interacting with the activated CD4 T cell (81). The B cell captures the native antigen by way of surface Ig and then internalizes it and processes it, placing peptides bound to its class II MHC molecules. The interaction with the T cell then takes place. Anatomically it is thought that it arises in the deep cortex next to the follicle. The APC/T cell interaction leads to reciprocal effects. These double effects are particularly pointed in the case of B cell/T cell interaction. The B cell is activated and differentiates while the T cell likewise continues on its activation pathway. The result is the dual recognition and specificity: a B cell that makes antibody reactive to the native protein antigen and the T cell that reacts with the peptides derived from intracellular processing.

Following these first interactions, the cellular interactions move to the follicles (81). There the follicular dendritic cell (FDC) traps the soluble antigen molecules as an antigen-antibody-complement complex. In the follicle, the B cells undergo extensive proliferation with differentiation. It is at this site where class switch and affinity maturation take place. Presumably the B cells are interacting with the FDC-bound complex in the context also of receiving signals from the activated T cells.

The Discrimination Between Self and Nonself by the Immune System

The molecular mechanisms that generate a diverse BCR and TCR library do not discriminate between receptors to foreign antigens or to self-proteins. Autoreactive cells do develop and have the potential to produce the autoimmune diseases. This is the price that we pay for having a vast repertoire of receptors. Actually, autoimmunity was first mentioned by Ehrlich using the term "horror autotoxicus" to describe what he foresaw as a disastrous event for the individual. After much debate, it is now well established that these conditions do take place and constitute a serious cause, or at least a component, of many diseases. Indeed, many if not all organs can be the spontaneous targets of autoimmunity in humans or can be induced targets in experimental animals strongly immunized with autologous antigens. The spontaneous human diseases with an autoimmune component include hemolytic anemias and thrombocytopenias, type I or insulin-dependent diabetes mellitus, lupus erythematosus, rheumatoid arthritis, some forms of thyroiditis, myasthenia gravis, and multiple sclerosis. But these are disease states because the normal immune system has developed mechanisms to control either the development or the activation of the autoreactive clones.

Several points stand out for explaining the control of self-reactivity, a subject of much research at present. As initially theorized by Joshua Lederberg (82), in the development of lymphocytes, the immature cells pass through a stage in which they become highly sensitive to the engagement of their receptors by antigen and die if this engagement takes place (Fig. 1-1). Thus, many autoreactive cells are deleted by a process called clonal anergy that involves the death of the cells by apoptosis. Much attention has now been given to how the differentiation of the early T and B cells takes place and how the selection of the cells reactive to foreign antigen (called positive selection) and those reactive to autologous antigen (called negative selection) takes place (83–87). In the former case, the cells live to form part of the peripheral lymphocyte pool that permits the species to confront the environment. In the latter case, the cells die and autoreactivity to some antigens is avoided. The differentiation of T cells and B cells has common and also distinct features that we now address in the context of analyzing self-reactivity.

The thymus gland is the primary lymphoid organ in which stem cells differentiate to thymocytes. This differentiation takes place most vigorously during fetal life and in early postnatal development. The essential role of the thymus was first noted by Jacques Miller: he thymectomized mice immediately after birth and before there was an opportunity for seeding of the secondary lymphoid organs (88,89). While thymectomy of the adult did not result in impairment of immune response, the mice thymectomized at birth were T-cell and immune deficient. [Birds also have a defined organ for B-cell development, the bursa of Fabricius (90); in mammals, however, B cells develop mostly in bone marrow. It follows that birds show with great distinction the pathways of B- and T-cell differentiation: bursectomy results in selective loss of B cells but with a healthy T-cell system, while in contrast thymectomy impairs selectively the T-cell development (91).]

The early T cell goes through defined stages as it moves in very close contact through the gland by means of a network of epithelial cells and dendritic cells that express class II MHC molecules. The differentiation can be tracked by following the expression of the CD4 and CD8 coreceptors. From the "double negative" stage the cells develop both coreceptors (the "double positive" stage) and then differentiate to either CD4 or CD8 T cells, some of which emigrate to constitute the peripheral population. At the double negative stage, the cells express the RAG enzymes and undergo recombination of their V, D, and J genes that encode the TCR beta chain, through an order rearrangement, first of the D and J gene segments and then with a V gene.

cytosis of antigen-antibody complexes by the antibody-forming cells was the stimulus for the production of additional antibodies (7). Various theories were proposed to account for this phenomenon, but there appeared to be no way of testing the hypotheses. Sir McFarland Burnet succinctly stated the problem confronting immunology in 1959 (9): "Until such methods of handling pure clones of cells in vitro are available, a choice between the clonal selection hypothesis of antibody formation and other alternatives can only be on the basis of convenience and their heuristic value in stimulating new experiments."

Cellular Immunology

Consequently, investigators focused on identifying and studying the cells comprising the immune system for the next 2 decades, thereby creating the science of cellular immunology. Nowell pioneered this new science by reporting in 1960 that lymphocytes are capable of proliferating in response to stimulation by mitogens (10), and his discovery was soon followed by the demonstration that specific antigens could also activate the proliferative expansion of antigen-selected cells. Prior to this discovery it had been thought that lymphocytes were end-stage cells, incapable of self-renewal, and they were not thought to be the source of the antibody-forming plasma cells. This understanding allowed Burnet's clonal selection hypothesis to be modified to include the proliferative expansion of the clones of antigen-specific cells after they had been selected. Then, in 1963 Jerne and Nordin introduced an assay capable of detecting individual antibody-forming cells, and for the first time the selection of antigen-specific cells, as well as their proliferative expansion, was visualized and quantified (11).

Subsequently, in 1967 Dutton and Mishell improved on Jerne's assay for antibody-forming cells by performing the entire immune response in vitro (12). This development was critical, as it permitted a reductionist approach to both the activation phase, as well as the effector phase, of the immune response for the first time. Armed with these new cellular assays, it became recognized that there were at least three distinct types of cells comprising the immune system, namely, B cells, T cells, and macrophages. Although plasma cells were found to be the actual antibody-forming cells, it was not shown that B cells are the precursors of plasma cells until 1970 when B cells were found to express immunoglobulin molecules on the cell surface (13,14). However, by 1970 it was realized that antibody formation by B cells/plasma cells depended on help derived from T cells and macrophages, although the mechanisms responsible for this help remained obscure (15).

The interdependence of the cells in generating an antibody response was further confounded by the observations of Benacerraf and coworkers, who reported that antibody production was genetically determined and that the cellular immune response was somehow related to this genetic linkage (16,17). Subsequently, McDevitt and coworkers reported that the magnitude of antibody production reactive with synthetic peptide antigens depended on the genes encoded by the major histocompatability complex (MHC) (18,19). Together, Benacerraf and McDevitt demonstrated that this immune response (Ir) gene effect depended on T-cell recognition of antigen (20).

Throughout the 1970s the process by which B cells, T cells, and macrophages cooperated to generate the production of antibodies remained a mystery. However, between 1965 and 1975 many investigators noted activities in the culture medium of proliferating lymphocytes that promote in vitro immune responses. Depending on the assays used to detect the activities, these functions augment either the generation of antibody-forming cells, the so-called T-cell replacing factors (TRF) (21), or the proliferation of T cells, the so-called blastogenic factors (BF) (22,23). However, the exact chemical nature of these activities remained obscure. As well, it was unclear whether these activities were required for the generation of an immune response or whether they merely served to amplify the response. Moreover, it remained controversial whether there were many such factors or whether one or a few molecules were responsible for all of the activities detected. In addition, the cellular sources of these various activities were equally mysterious, as both T cells (24) and macrophages (25,26) were implicated.

Assays that detected the capacity of T cells to kill target cells through direct cell-cell contact were devised in 1968 by Brunner et al (27). Subsequently, Zinkernagel and Doherty reported in 1974 that cytotoxic T lymphocytes (CTL) could only recognize specific antigens if expressed by histocompatible target cells (28), a phenomenon that came to be known as MHC restriction of CTL antigen recognition. This finding and the previous observations regarding the MHC restriction of antibody production led to an explosive controversy, concerning the nature of the T-cell antigen receptor (TCR) and how it recognized both antigen and MHC-encoded molecules, that continued unabated for the next decade.

In 1975 Rolf Keissling, Eva Klein, and Hans Wigzell and, independently, Ronald Herberman's group discovered a different type of killer cell that did not appear to recognize specific antigens or to be restricted by the MHC. This cell became known as the natural killer (NK) cell, which was thought to be analogous to the natural antibodies that were found in individuals in the absence of specific immunization. The origin of NK cells remained controversial for more than 2 decades; however, with additional methods for identifying B cells and T cells, it became known that NK cells represent a distinct lineage of lymphocytes.

Also in 1975 Kohler and Milstein made the surprising discovery that somatic-cell hybrids between immunized splenic B cells and murine plasmacytoma cell lines could make continuous quantities of monoclonal antibodies (MoAbs) (29). Thus, Burnet's clonal selection hypothesis was finally proved correct, and his prediction that the proof would rest with the ability to manipulate and study the clonal progeny of a single cell was borne out. The discovery of hybridomas and MoAbs revived the idea of using antibodies as therapeutic "magic bullets." However, the technology to make human monoclonal antibodies proved difficult, and the human antimouse antibody (HAMA) response, which is really a form of serum sickness, effectively precluded the widespread use of mouse MoAbs as therapeutic agents.

In 1976 Morgan and Ruscetti working in Robert Gallo's laboratory reported that culture medium conditioned by proliferating lymphocytes promoted the long-term growth of T cells (30). Subsequently, using conditioned medium containing the T-cell growth factor (TCGF) activity, we reported the creation of the first, antigen-specific T-cell clones in 1979, 20 years after Burnet first forwarded his clonal selection hypothesis (31). Having reduced the tremendous diversity of antigen recognition by the cell population to the progeny of a single cell, it was then possible to prove that antigen recognition by T cells, like B cells, was also clonal. At the time, we predicted that monoclonal T cells would be just as important for future studies directed at determining the function of T cells as monoclonal antibody-producing cells were for understanding the function of B cells. Thus, we felt that "the growth of large numbers of monoclonal antigen-specific T cells would lead to the identification and molecular characterization of the TCR, the mechanisms responsible for T cell cytolysis, and the identification of T cell differentiation markers" (31).

Monoclonal antibodies reactive with distinct, human T-cell subsets were generated at the same time that we had generated T-cell clones (32), and in a series of reports Reinherz et al demonstrated that these antibodies were useful in defining helper (CD4+) and cytotoxic (CD8+) T cells (33). Subsequently, they discovered that an antibody that recognized a molecule expressed on all peripheral T cells (CD3) blocked T-cell proliferation in response to mitogens, while this same MoAb was mitogenic itself (34).

Molecular Immunology

While the 1960s and 1970s were devoted to identifying and isolating the cells responsible for the immune response, the 1980s and 1990s were devoted to discovering and characterizing the molecules involved in promoting the proliferation and differentiation of the various cells, particularly the T cells. With TCGF-dependent T-cell clones available, we created a quantitative assay for the TCGF activity (35), which we then used together with standard biochemical methods to identify, characterize, and purify the molecule responsible for the activity (36). Subsequently, we generated monoclonal antibodies reactive with the purified molecule, and then used these antibodies to purify the molecule to homogeneity (37). These findings were also the first to show that a lymphokine or cytokine *activity* could be ascribed to a single molecule and not several molecules, as had been proposed by others. Moreover, these findings directed us to a series of experiments that eventually identified the first cytokine receptor (38) and to the conclusion that cytokines function in exactly the same way as classic hormones, that is, by binding with high affinity to specific cell-surface receptors.

Anticipating the discovery of additional cytokines, in 1979 this new class of molecules was named interleukins, to designate that they functioned to signal *between leukocytes*. At that time, an activity derived from macrophages had been identified that we had shown functioned to augment TCGF production by T cells (39,40). Therefore, the macrophage product, which had been termed lymphocyte-activating factor, was renamed interleukin-1 (IL-1), and the TCGF molecule was renamed interleukin-2 (IL-2).

The decade of the 1980s produced an exponential increase in the amount of information available regarding the molecules of the immune system and in our understanding of how those molecules function to initiate and regulate the immune reaction, all of which set the stage for immunostimulatory therapy. Using antigen-specific T-cell clones and hybridomas and clone-specific monoclonal antibodies, the T-cell antigen receptor (TCR) was identified and characterized biochemically by Meuer et al (41,42) and by Haskins et al (43). Soon thereafter, Hedrick et al isolated the first cDNA encoding one of the four chains of the receptor (44). This information placed the TCR into the immunoglobulin superfamily and revealed that the basic structures of the TCR and immunoglobulins are quite similar. Subsequent studies focused on TCR signaling and gene activation revealed that TCR triggering is obligatory for the transcriptional activation of the IL-2 gene (45–47), as well as of the genes encoding the IL-2 receptor (IL-2R) (48–50). Subsequently, the same TCR triggering was found to be responsible for the expression of additional cytokines as they came to be discovered.

Dendritic cells were discovered and found to present antigen to T cells with marked efficiency (51). Moreover, B cells were also shown to present antigen effectively to T cells (52). Also, macrophages were found to process protein antigens and present peptides as antigens to the TCR, revealing that T cells recognized fundamentally different antigenic molecules as compared with antibodies (53). Ultimately, the structure of MHC-encoded molecules was determined, and for the first time it was realized how antigenic peptides are bound to the MHC molecules and presented to T cells, thereby solving the controversy of MHC restriction of TCR antigen recognition (54). The TCR recognizes a complex of peptide antigen and MHC-encoded molecules. This revelation explained the MHC restriction of immune responses, which is that CD4+ helper T cells recognize antigens bound to MHC class II–encoded molecules and that CD8+ CTL recognizes antigens bound to MHC class I–encoded molecules.

During the 1980s and 90s, almost two dozen new interleukins were discovered, most of them with homologies to IL-2. Some of these interleukins such as IL-4 (55), IL-6 (56), and IL-10 (57) were found to be important in the production of antibodies. Therefore, the T-cell replacing factor activity described in the early 1970s was explained in part by the discovery and characterization of these cytokines. Direct cell-cell contact between helper T cells and B cells, mediated by T-cell–derived CD40 ligand and B-cell CD40, was also demonstrated to be necessary for optimal T-cell help in antibody formation (58). Other cytokines, in particular IFN-γ (59) and IL-12 (60), were found to be crucial to the generation of classic, cell-mediated, immune responses such as the delayed type hypersensitivity (DTH) reaction. IL-2 was found to be important for the production and action of both the so-called TH-1 cytokines (i.e., DTH cytokines) and the TH-2 cytokines (i.e., antibody-related cytokines) (61,62). IL-2 became the first interleukin to be resolved at the genetic level by Taniguchi et al (63), and recombinant DNA technology

of approaches to engineer HIV reactivity in nonspecifically expanded cells by the introduction of chimeric receptors that can recognize infected targets. Receptors containing extracellular domains composed of either the variable region of an envelope-specific antibody or the CD4 molecule fused to the cytoplasmic signaling domain of the TCR zeta chain have been expressed in T cells and shown to bind and kill HIV-infected targets in vitro (195,196). Adoptive transfer of these genetically modified cytolytic cells may need to overcome the problem posed by the large, cell-free HIV burden in some patients that may occupy the receptor, and it is unclear if repetitive triggering through these chimeric receptors in vivo will be sufficient to result in retention of cytolytic activity or permit cell survival. Clinical trials are being conducted in HIV-infected patients to assess these issues and should provide directions for developing this cellular therapy regimen.

Adoptive T-Cell Therapy of Viral Diseases: Future Potential

The adoptive transfer of virus-specific T cells is emerging as a useful approach for the prophylaxis or treatment of CMV and EBV infections in immunodeficient recipients of allogeneic HCT. Several limitations of this approach have been identified and will need to be addressed in future studies. One is the susceptibility of transferred T cells to the inhibitory effects of the intensive immunosuppressive drug therapy that is often being concurrently administered to these patients. This could potentially be overcome by genetically modifying the transferred T cells to be resistant to one or more of the immunosuppressive drugs. Strategies for rendering cells resistant to cyclosporin by expressing a mutant calcineurin protein have been described (197), and similar approaches are being pursued for other commonly used agents. A second issue, related to the use of T-cell therapy for CMV and EBV in solid-organ transplant patients who develop a primary infection from the transplanted organ, is the difficulty isolating T cells from the recipient for use in therapy. This obstacle could potentially be resolved by introducing the TCR α and β genes derived from a T-cell clone of known antigen specificity and MHC restriction into T cells from the recipient to confer the desired reactivity (198).

Reservoirs of replicating and latent HIV persist in infected individuals despite combination antiretroviral drug therapy, and maintaining HIV-specific T-cell responses by adoptive transfer could assist in reducing these viral reservoirs. However, as discussed above, it may be necessary to genetically modify $CD8^+$ T cells to overcome limitations imposed by the $CD4^+$ Th deficiency in these individuals, and it will be essential to ensure that T cells can traffic to the tissue sites containing reservoirs of HIV.

Viruses such as EBV, HPV, and KSHV have been associated with the development of malignancies in humans, and an emerging area of interest is to augment T-cell responses to viral proteins expressed in tumor cells by vaccination or T-cell therapy. This issue will be discussed more extensively in the next section. However, a more complete understanding of the mechanisms these tumors use to evade recognition, despite the expression of immunogenic proteins, is likely to be necessary to design appropriate therapeutic strategies.

Adoptive Cellular Therapy of Tumors

Cellular therapy has been more extensively pursued for the treatment of human malignancy than for human viral diseases. This has in part reflected the intense need to develop new treatment strategies for cancer and has resulted in testing not only specific T cells but the more readily generated nonspecific effector-cell populations that can lyse tumor cells in vitro and that also have in vivo activity in animal tumor models. However, the initial human clinical trials, particularly with nonspecific effector cells, have had limited success, and the substantial obstacles to effectively and broadly translating this strategy to human tumor therapy must be resolved before the potential demonstrated in murine models can be realized. These obstacles appear more profound than those previously described for developing therapy for viral infections, as a result of both the failure of most tumors to encode readily characterizable novel antigens that induce strong T-cell responses and the more complex biology of tumors as compared with viruses. However, recent advances in cellular and molecular immunology have provided approaches for identifying target antigens at which to direct an immunologic attack, as well as insights into the underlying biology. Thus, it can be expected that cellular therapy using specific tumor-reactive T cells will become increasingly available and hopefully effective for the treatment of a wide range of malignancies.

Effector Cells Capable of Mediating Antitumor Responses

Adoptive transfer studies in mice have been informative for examining the operative in vivo effector mechanisms and contributions to tumor eradication of cell populations that exhibit antitumor activity in vitro. Several cell types with distinct modes of tumor cell recognition have been shown to promote the regression of established tumors in syngeneic hosts, including nonspecific effector cells (such as NK cells, lymphokine-activated killer (LAK) cells, and activated monocytes) and MHC-restricted $CD4^+$ and $CD8^+$ tumor-reactive T cells (88,199–203). Although the relative ease with which nonspecific effectors can be generated makes such cells attractive for use in adoptive therapy, even in animal models the in vivo activity of these cell populations has been limited and often restricted to localized tumors. The clinical trials performed in human cancer patients with nonspecific effector cells have provided provocative but ultimately largely disappointing results (204–206), and no clear strategies are available to overcome the lack of tumor specificity and inability to selectively localize at sites of tumor. Thus, since $CD4^+$ and $CD8^+$ tumor-reactive T cells exhibit specificity for target antigens expressed by tumor cells, homing to sites of antigen expression and the most significant and reproducible in vivo antitumor activity in murine models, this chapter will focus on the development

of cellular therapy with $\alpha\beta^+$ T cells. Although most of these T cells recognize proteins in the context of classic MHC antigens, reports of T cells recognizing tumors by non-classic MHC molecules are appearing (207,208).

Class I–restricted $CD8^+$ tumor-specific CTL directly lyse class I–positive tumor cells in vitro, and adoptively transferred $CD8^+$ CTL mediate regression of a wide range of experimental tumors in vivo (88,209–211). The effector mechanisms employed by $CD8^+$ T cells extend beyond direct cytolysis and include the antitumor activities of secreted cytokines (212). Since the majority of tumors express or can be induced to express class I molecules, $CD8^+$ CTL represent the effector cell of choice for adoptive transfer in most therapy settings. However, as the elimination of established tumors generally requires a prolonged in vivo antitumor response (213), the therapeutic efficacy of transferred $CD8^+$ CTL is influenced by the availability of cytokines normally provided in an immune response by $CD4^+$ T cells such as IL-2, which is required to promote proliferation and survival of these effector cells. Thus, it has generally been necessary in murine models either to administer IL-2 or to concurrently transfer tumor-reactive $CD4^+$ T cells to achieve tumor eradication (88,209,211,214,215). As an exception, $CD8^+$ CTL that produce IL-2 in response to tumor recognition can be occasionally isolated from mice. The improved in vivo efficacy observed in tumor therapy with such bifunctional, helper-independent $CD8^+$ CTL has also provided direct support for the critical need to provide IL-2 in tumor therapy, if the more classic IL-2–dependent $CD8^+$ CTL are being administered alone (216).

Class II–restricted $CD4^+$ T cells, in addition to providing helper function for $CD8^+$ T cells by providing IL-2 and/or by activating professional antigen-presenting cells to effectively stimulate $CD8^+$ cells (217–219), can independently mediate antitumor effects (220,221). For class II^+ tumors such as B-cell lymphomas, $CD4^+$ T cells may directly lyse the target cells (222,223). Moreover, studies in murine models with a wide range of histologic tumor types have demonstrated that noncytolytic $CD4^+$ T cells can also mediate rejection of class II^- tumors in the absence of participation by $CD8^+$ T cells (201,202,224). This presumably requires class II^+ APC to present tumor antigens to stimulate the $CD4^+$ T cells to secrete cytokines that are directly tumoricidal; to recruit and activate tumoricidal effector cells such as NK cells, macrophages, and eosinophils; and/or to interfere with tumor angiogenesis (225). Unfortunately, these studies in animal models have not identified the settings in which $CD4^+$ T cells that recognize a tumor antigen can be predicted to be effective in the absence of $CD8^+$ T cells, or in which the effector responses promoted by $CD4^+$ T cells provide an obligate contribution to the efficacy of $CD8^+$ Tc. Until such principles are defined, some human tumor therapy trials using only tumor-reactive $CD4^+$ T cells or only $CD8^+$ T cells may be unsuccessful and may underestimate the potential antitumor activity of the infused T-cell population.

Molecular strategies have now provided the means to generate a new population of hybrid effector cells—cytolytic effector T cells into which have been inserted, by gene transfer with retroviruses or alternative vectors, a chimeric receptor with an extracellular binding domain derived from a tumor-reactive antibody and a cytoplasmic signaling domain derived from the zeta chain (or a functional surrogate) of the T-cell receptor (226). By molecular engineering, it is possible to construct this entire receptor from one gene encoding a single chain containing the antibody V_H and V_L regions separated by a flexible linker that permits formation of the high-affinity antibody-binding site fused inframe to the signaling tail. Effector $CD8^+$ cells expressing these "T-body" receptors have been shown to recognize and lyse tumor cells and to eradicate tumors in in vivo animal models (226–228). This approach has the obvious advantages of greatly broadening the range of antigens that can serve as targets for therapy and of circumventing some of the immune evasion strategies employed by tumors, while still utilizing the effector mechanisms mediated by T cells. However, there are several limitations that must be addressed. Firstly, the receptor could potentially be occupied/triggered by soluble antigen, which would prevent therapeutic efficacy. Secondly, the receptor affinities and target interactions may preclude the necessary kinetics of binding and release required for normal, T-cell function and survival. Thirdly, the inability of these receptors to assemble a complete TCR complex and to engage all the appropriate accessory molecules on target recognition could attenuate T-cell function. Because of these differences between the T-body receptor and a normal TCR, there is only limited evidence to suggest that these effector cells can mediate serial engagement and killing of targets and antigen-induced proliferation (229), which is required to sustain a prolonged in vivo response. Ongoing, basic, preclinical and clinical investigations to improve receptor functioning and signaling offer significant promise (230,231), however, and may provide substantial benefit in the future.

Identification of Tumor Antigens Recognized by T Cells

In contrast to viral diseases and experimental murine tumors that express novel antigens and readily elicit T-cell responses that can be examined for therapeutic efficacy, initial studies with human tumors failed to detect T-cell responses in the majority of tumor-bearing hosts. Thus, a major obstacle to developing T-cell therapy for human malignancy has been the absence of defined antigens expressed by tumors to be appropriate targets for an immunologic attack. In the past decade, considerable effort has been devoted to the identification of human tumor antigens, and the success of these efforts has now provided the foundation for developing cellular therapy of human malignancy using T cells specific for defined tumor antigens.

Three strategies, initially used to identify proteins in murine tumor cells that give rise to antigenic peptides recognized by $CD8^+$ or $CD4^+$ T cells, are now being used to study human tumors. The first approach, pioneered by Thierry Boon et al, has used tumor-reactive Tc as the reagents to identify immunogenic proteins. Pools of DNA from an immunogenic murine P815 tumor cell were transfected into target cells, and the targets screened for recognition by tumor-reactive Tc clones (232). Using this strat-

Immunology

Therapeutic Immunology, Second Edition
K. F. Austen, S. J. Burakoff, F.S. Rosen, T. B. Strom, Editors

The second edition of *Therapeutic Immunology* provides a thorough and current review of existing and emerging treatments for diseases of immunologic origin with 14 new chapters and 36 new contributors. Editors and contributors who are recognized leaders in their specialties provide current information on the full spectrum of topics related to intervention, including antibodies, cytokines, gene and cell therapies, and vaccines.

The book should prove a valuable reference for all researchers and physicians working in the field of immunologic disease. Practicing clinicians will appreciate the authors' emphasis on the underlying principles behind clinical interventions and discussions of the applications of current research in immunology, molecular biology, and biotechnology. Three sections covering drugs, biologic agents, and maneuvers demonstrate how the latest developments in immunology and biotechnology can provide treatment strategies for many previously incurable diseases.

Related Titles

New Concepts in the Immunopathogenesis of CNS Infections
P. K. Peterson and J. S. Remington, Editors, 0-632-04528-0

Pathology and Immunology of Transplantation and Rejection
S. Thiru and H. Waldmen, Editors, 0-632-03676-1

Immunosuppression
L. C. Ginns, A. Benedict Cosimi, P. J. Morris, Editors, 0-632-04445-4

Clinical Diagnostic Immunology
R. Nakamura and J. Folds, Editors, 0-86542-518-3

Visit us online!
blackwellscience.com
books | journals | e-media

Blackwell Science

Cover design and illustration by
Hans Neuhart, Electronic Illustrators Group Eig.net

ISBN 0-632-04359-8
90000>
9 780632 043590